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13. ABSTRACT (Maximum 200 Words)

Bone fluid flow is hypothesized to initiate aberrant remodeling which can ultimately compromise bone quantity and quality. Thus, pathologic response of load-induced fluid flow can potentially damage tissue viability, and initiate bone's remodeling process, ultimately leading to the stress fracture syndrome. Results from this year's study have shown that fluid flow stimuli in bone has potentials not only in initiating bone's remodeling, but also in vasculature adaptation. Within the physiological range, bone formation is proportional to applied fluid flow stimulation. While the pressure exceeds the physiological intensity or falls within the pathologic range, i.e., addition to normal physical loading, it triggers extensive remodeling and thus even weaken the quality of bone, i.e., increase intracortical porosity and induce lesions. Repetitive cyclic fluid flow in bone has shown an effect on the nutrient vessel remodeling inducing vessel wall thickening and smooth muscle cell proliferation, which may potentially contribute to pathological remodeling in bone. These results may provide insight of fluid flow in physiological and pathological remodeling and its role in stress fracture.

To understand the etiologic factors of stress fracture is extremely important. As a short-term goal, this study is aimed to acquire an improved understanding of the patho-physiology of stress fractures at the tissue level such as fluid flow alone triggers the osteopenia and lesion. As a long-range goal, if we can identify these osteogenic signals, this may help to design or alter specific training regimes to reduce the risk factors of stress fractures.

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A. Introduction

It is widely accepted that stress fractures are the result of accumulated fatigue microdamage, yet there several other candidate mechanical factors could – as likely - contribute the deterioration of the bone tissue. Load-induced interstitial fluid flow, a primary regulator of bone adaptive responses, could initiate the stress fracture syndrome by upregulating the resorption of the cortex, independent of a repair process catalyzed by material corruption.

Stress fractures almost exclusively occur in physically active individuals, e.g., dancers, joggers, and soldiers, in a variety of skeletal location but mostly in lower limb, e.g., femur, tibia, and calcaneus [2]. The impact of stress fractures is severe, which 70% of all stress fractures are reported in runners and is ranked as the most common risk to running [4].

Preliminary data from our lab has shown that intracortical fluid flow can be induced not only by bone matrix strain, but by the intramedullary pressure (ImP) generated during loading. Increased ImP pressures arising from redundant axial loading in turn severely compromises perfusion of the bone tissue, and thus alters the vascular fluid supply. Thus, pathologic levels of load-induced flow can damage tissue viability, and thus initiate bone's remodeling process, ultimately leading to the stress fracture syndrome. Importantly, alterations in ImP fluid pressure can stimulate remodeling even under conditions of minimal bone strain, and thus pathology can arise even in the absence of intense physical loading of the bone tissue. In the work proposed, we hypothesize that:

Persistent levels of bone fluid flow, physiologic in magnitude, will initiate aberrant remodeling which ultimately compromises bone quantity and quality.

The technical objectives of this research is to evaluate 1) repetitive fluid flow, as dependent on magnitude and duration, stimulates pathological remodeling and ultimately compromise the material properties of the bone; and 2) cyclic intramedullary pressure can alter the nutrient vessel blood supply and partially reduce nutritional flow to the cortex.

The scope of this project is focused on the etiological mechanism of stress fracture induced by load-generated interstitial fluid flow within the matrix. It is proposed that mechanical loading results in deformation of bone matrix and the substantial interstitial fluid space, which generates pressure gradients and further induces interstitial fluid flow [6]. Cortical bone is composed of a solid matrix phase and an interstitial fluid phase [7,1,5,9,8,11], which may trigger bone cell sensing, signaling and responding to physical stimuli, as well as nutrient transport. This intracortical fluid flow is considered a critical mediator of bone mass and morphology [3,5,9,8,10]. Fluid flow may represent a critical role to explain strain magnitude, strain rate and gradient regulated bone formation, remodeling, and weakening of bone.

Fluid flow as a pathogenic factor contributing to aberrant remodeling may depend on the magnitude of stimulus, i.e., hydraulic fatigue, and triggered by alteration of nutrients supply. In this study, the results have shown that low magnitudes of ImP could initiate spatial fluid flow in bone and thus stimulate bone adaptive response. Within the physiological range, new bone formation is proportional to applied fluid flow stimulation. While the pressure applied exceeds the physiological intensity or falls within the pathologic range, i.e., addition to normal physical loading, it may trigger extensive remodeling and thus even weaken the quality of bone, i.e., increase intracortical porosity and induce lesions. This also suggests that oscillation of ImP may influence the perfusion of blood supply in bone, which has potential to generate nutrient vessel remodeling. This can result in the reduction of the blood supply to bone, and further result in pathological remodeling and weaken the bone mass. These results may provide promising evidences that bone fluid flow is likely an etiology factor initiating bone remodeling and

substantial stress related fractures.

B. Research accomplishments

In this study, we hypothesize that persistent levels of bone fluid flow, physiologic in magnitude, can initiate aberrant remodeling which can ultimately compromise bone quantity and quality. Thus, pathologic response of load-induced fluid flow can potentially damage tissue viability, and initiate bone's remodeling process, ultimately leading to the stress fracture syndrome. The PI and the research team are grateful for the opportunity of this research grant from the USAMRMC. The preliminary results from this research may lead to a new insight in understanding fluid flow induced bone remodeling and its resultant stress fracture mechanism. The research goal was initially proposed to be achieved through two primary sub-hypotheses and specific aims: (1) repetitive fluid flow, as dependent on magnitude and duration, will stimulate pathological remodeling *in the absence of matrix strain* and ultimately compromise the material properties of the bone; and (2) cyclic intramedullary pressure (ImP) will cause nutrient vessel remodeling and constriction, and thus partially reduce nutritional flow to the cortex.
Results of the study demonstrate progressive achievements in the areas of (a) **repetitive, long duration, fluid pressure oscillatory loading can generate nutrient vessel wall and smooth cell remodeling, which potentially reduce blood flow in bone; (b)** dose dependence of bone remodeling elucidated by dynamic fluid flow stimulation in a disuse model; (c) trabecular bone adaptation induced by low intensity, high cycle number oscillatory intramedullary pressure stimulation in an intact model; (d) remodeling initiated by repetitive fluid flow serves as an etiologic factor for osteopenic lesions and stress fractures in an intact model; (e) long duration, repetitive, oscillatory fluid loading initiates the reduction of bone blood volume flow in nutrient vessels; and (f) repetitive, long duration, intramedullary fluid pressure oscillatory loading can generate nutrient vessel wall remodeling and potentially reduce blood flow in bone

(1) Repetitive, long duration, intramedullary fluid pressure oscillatory loading can generate nutrient vessel wall remodeling and potentially reduce blood flow in bone

Bone fluid flow induced by loading and intramedullary pressure has been demonstrated to mediate bone modeling in the absence of mechanical strain. To further evaluate the potential mechanism of this fluid flow effect on altering the nutrient supply, we hypothesize that fluid flow generated by ImP oscillation can generate nutrient vasculature adaptation which may serve as a source for bone modeling. Thus, our objective is to investigate the relationship between cyclic hydraulic stimulation in the marrow cavity and nutrient blood vessel adaptation.

Arterial vessel wall adaptation to acute or chronic flow changes is proposed to respond to dynamic fluid pressure and fluid shear stress at smooth muscle and the endothelium. Such vessel adaptation in the nutrient artery of bone can be potentially induced by mechanical load generated flow.

Using an avian model, cyclic hydraulic stimuli was applied to the left ulnae, 10 minutes per day, with magnitude of 50-90mmHg at 3Hz and 30Hz for 2, 3, & 4 weeks (n=32), while the right ulnae were left unloaded as sham control. Four additional birds were used as age-matched control, in which both left and right ulnae were unloaded. The adaptive responses of the nutrient vessels were analyzed through a standard soft tissue histology procedure. The histomorphometry of vessels were examined using a digitized microscope and computerized image processing. The average vessel wall thickness, lumen perimeter and smooth muscle cell (SMC) layer number were assessed. The lumen area was calculated from the measurement of lumen perimeter. Our data showed that the loaded side, increased in average vessel wall thickness and SMC layer number, yet decreased in lumen area. The results indicate that the cross-sectional vessel area

increases after high cycles of loading, contributed by vessel wall thickening and lumen aeral reduction. The area of the vessel increased as the duration of loading increased, i.e., vessel area for the 3-weeks of loading is 9% greater than the 2-weeks, and the area for the 4-weeks loading is 36% higher than the 3-weeks and 42% higher than the 2-weeks (Fig. 1). Loading at 3Hz, 70-90mmHg produced 10% increase in SMC layer number, while loading at 30Hz showed a 17% increase in SMC layer number (Fig. 2) with a decrease in lumen area, 31%. This result suggests that repetitive cyclic fluid loading in bone does have an effect on the nutrient vessel adaptation, which may further reduce the blood supply to bone and potentially generate pathological remodeling in bone tissue. This mechanism may partly contribute to certain skeletal diseases, e.g., stress fracture.

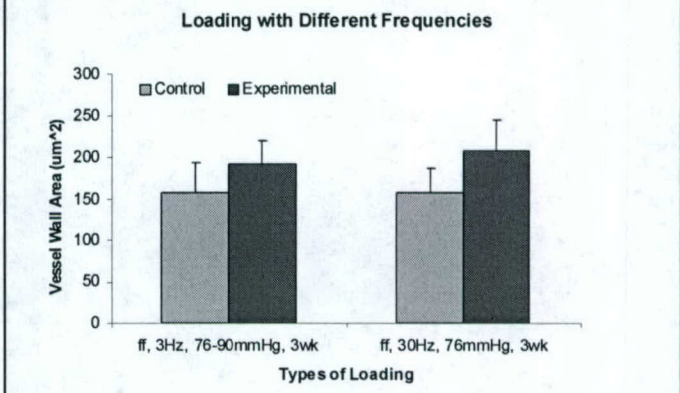

Fig. 1. Histomorphometry of the nutrient vessel cross sections analyzed from ulnae subjected to high repetitive fluid flow (ff) 10 min per day for 3 weeks at 3Hz (1800 cycles/day) and 30Hz (18000 cycle/day). High cycles of fluid flow results in vessel thickening.

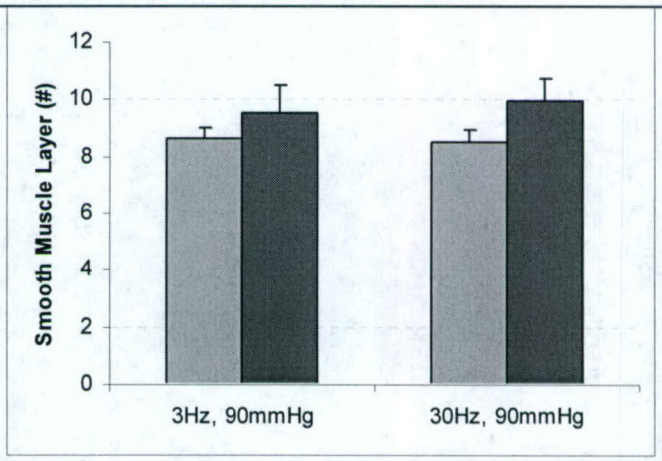

Fig. 2. ImP induced vessel cross-sectional smooth muscle cell layer change.

Implication of this work: The results imply that repetitive fluid flow loading may generate hyper-tension in the nutrient vessel and induce vessel wall thickening, and may substantially reduce the nutritional supply of bone.

(2) Dose dependence of bone formation and bone remodeling elucidated by dynamic fluid flow stimulation.

Bone's adaptive response to the dose of fluid flow stimulus was evaluated in this preliminary in vivo experiment using a single frequency of 30 Hz.

Experimental design: The left ulnae of 12 adult, one year old male turkeys were functionally isolated via transverse epiphyseal osteotomies (Fig. 3). A sinusoidal fluid pressure was applied to the ulna in the physiological range at 30 Hz, 10 min/day, for 4 weeks (Table 1).

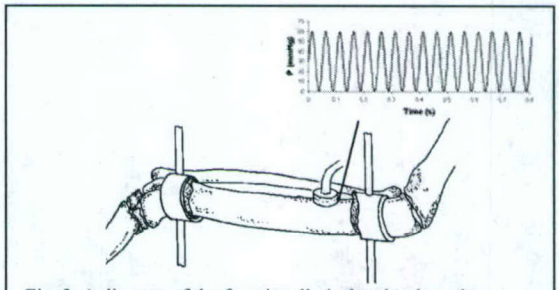

Fig. 3. A diagram of the functionally isolated turkey ulna preparation, such that oscillations in fluid flow can be achieved in the absence of matrix strain. External fixators on the two steel pins at two ends of ulna prevented external mechanical loads. A specially designed device placed into the marrow cavity achieved oscillatory fluid loading. Low magnitude (15 mmHg, 76 mmHg, and 105 mmHg) and high frequency (30 Hz) fluid pressure signal was imposed 10 min daily for 4 weeks in the disuse plus loading group, while the same procedures were prepared for disuse, yet the bone was subject to no exogenous fluid loading.

Table 1. ImP in a disuse model

ImP (mmHg)	N	Physiologic level
15	4	Marrow pressure generated by animal blood pressure
76	4	Close to 700 με peak strain induced marrow pressure
105	4	Pressure induced by bone impact exercise

The adaptive responses of bone were determined through morphometric measurement at the mid-diaphyses; the cortical area of each animal was compared to the contralateral control ulna. The histomorphometry was analyzed by calculating total area adaptation of periosteal and endosteal new surface bone (NB) formation and intracortical porosity using custom designed computer software. The ratio of net change of NB formation was determined by comparing NB and porosity to the area of original bone.

Results: All animals subjected to fluid flow loading showed a maintenance or gain of total bone mass. While NB formation at the endosteal surface showed no significant difference among applied pressures at 15, 76 and 105 mmHg, the periosteal surfaces did demonstrate dose sensitivity of NB formation. NB increased as a result of an increase in loading pressure, i.e., 2.4±0.3% at 15 mmHg, 5.0±2.0% at 76 mmHg and 8.4±3.7% at 105 mmHg (Fig. 4). Disuse resulted in approximately 3% intracortical porosity. These remodeling experiments have shown non-uniform spatial distribution at the endosteal and periosteal surfaces. Interestingly, increasing ImP did not inhibit intracortical porosity, but rather activated remodeling in the cortex (Fig. 4).

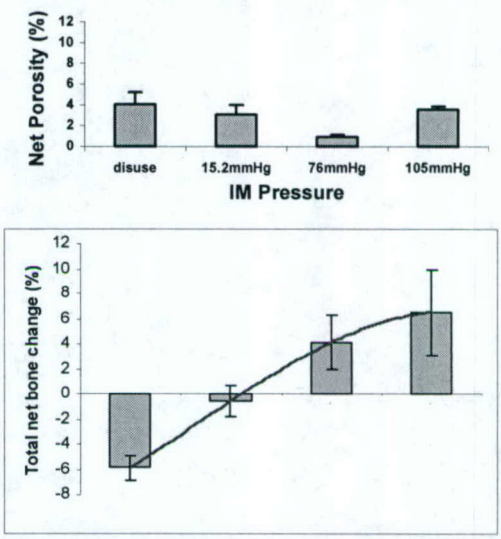

Fig. 4a (top). 4-week disuse resulted in significant intracortical porosity. Fluid flow stimulation inhibited such bone resorption process only at specific pressure magnitude, i.e., 60~80 mmHg.
Fig. 4b (bottom). Histomorphometry of a 100 μm section of the midshaft of ulna analyzed from bones subjected to the disuse control, and ImP stimuli at 15, 76 and 105 mmHg, 10 min per day for 4 weeks. Total new bone calculated by endosteal and periosteal new bone formation, indicated increase as increase of physiological ImP.

Discussion: These data confirm that fluid flow can significantly elucidate adaptive response if applied at proper fluid pressures and cycles or frequency. The results demonstrate that low magnitudes of ImP initiate spatial fluid flow in bone and thus stimulate bone's adaptive response. This suggests that oscillation of ImP can influence the perfusion of bone tissue in many ways. For example, at low physiological levels, ImP induced by circulation alone is on the order of 18 mmHg (2.38 kPa), which will provide basic nutritional supply and fluid pressure gradients to the bone. Resting or inactive of ImP, (i.e., aging, bed rest and microgravity) will influence the fluid perfusion in bone and may substantially stimulate remodeling. At high physiological magnitude, ImP can increase and enhance this perfusion process through increasing fluid pressure. When applied pressure exceeds the physiological intensity or is in the pathologic range, it will trigger an extensive remodeling process and even weaken the quality of bone.

Implication of this work: Loading induced physiologic fluid flow applied to a disuse model can serve as a mediator for increasing both new bone formation and intracortical porosities dependent on the magnitude of the fluid pressure and loading cycles. These experiments may yield new insights into the mechanism, at least at the tissue level, by which dose of bone fluid flow initiates and controls bone morphology and lead to a proper definition of anabolic fluid flow magnitude.

(3) Dynamic ImP induced trabecular bone adaptation

It has been demonstrated that load-induced fluid flow significantly mediates bone mass and morphology in the cortical region. While the fluid stimulus can be controlled quantitatively and potentially applied therapeutically in promoting turnover, the hypothesis of fluid induced

trabecular bone remodeling was evaluated in an intact avian ulna model using fluid loading while the animal kept up normal activities on the bone (Fig. 5).

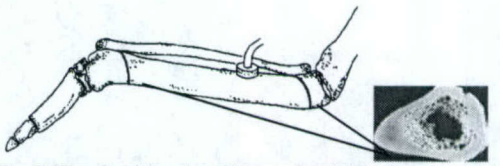

Fig.5. The fluid loader allows a variable frequency and fluid amplitude to be applied inside the bone. The fluid flow was applied to bone in addition to animals' normal activity. Trabecular sections were taken from proximal and distal ends of the ulnae (curved lines), and histomorphometric analysis was used for determining bone's adaptation.

Experimental protocol: The experimental ulna was fluid flow loaded with a pulsatile fluid pressure applied inside the canal at specific frequencies. Three experimental groups were included:
Group A: 30 Hz, 76 mmHg, 10 min/day, 4 weeks (n=3); Group B: 1 Hz, 76 mmHg, 10 min/day, 4 weeks (n=4); Group C: 30 Hz, 10 mmHg, 10 min/day, 4 weeks (n=4).On the contralateral ulna, a dummy device was connected into the bone with the same surgical procedure for the sham control. The trabecular regions at both proximal and distal ends were analyzed using histomorphometric measurement (Osteomeasure software, GA) (Fig. 6).

Results: The results reveal an increase of 22.7%±7.2 in trabecular volume [New Bone Volume / Bone Volume (BV/TV)] ($p<0.05$) for group A (30 Hz, 76mmHg) (Fig.7). BV/TV in Group B (1Hz, 76 mmHg) had only 0.5 % increase between loaded and control bones with no significance. Low magnitude, high frequency, fluid stimulation (Group C, 30 Hz, 10 mmHg) increased BV/TV (15.7%±7.4, $p<0.05$) (Fig. 7).

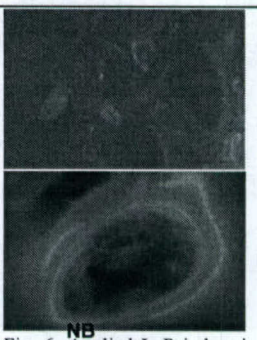

Fig. 6. Applied ImP induced trabecular bone modeling through fluorescent labeling morphometric analysis. Fluid loading generated 23% bone formation (NB) (bottom), while sham control indicate 2.5% new bone formation (above).

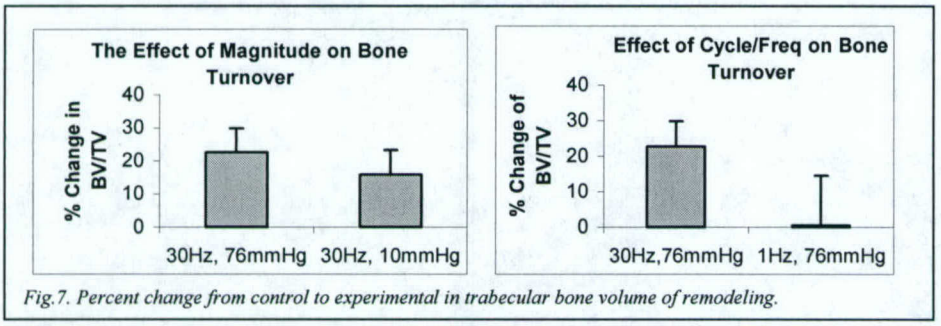

Fig.7. Percent change from control to experimental in trabecular bone volume of remodeling.

Discussion: The data demonstrate that a low magnitude of fluid flow can initiate a sufficient signal for bone's adaptive response in the trabecular region if applied at a proper duration and cycles (via higher frequency). When applying similar physiologic fluid pressure (i.e., 76 mmHg), a high cycle number (18k) of fluid stimuli generate much higher remodeling response (23% bone volume change) than a lower cycle number loading (0.6k) (0.5% BV/TV change). Interestingly, those bones loaded at the high cycle numbers, with smaller fluid magnitude perturbation (i.e., 18K cycles with 10 mmHg) has shown significant bone turnover in the trabecular region (i.e., 16% change of BV/TV). This implies that bone turnover may be more sensitive to the flow cycle number (elevated by frequency) than the pressure amplitude. These data suggest that high cycle of repetitive flow stimuli indeed have strong influence on adaptive process. This may influence fluid perfusion, convection, and surface fluid shear stress.

Implication of this work: This data suggests that fluid flow may, in fact, be the cause of bone remodeling from environmental strain. High cycles of repetitive flow stimuli can have a strong influence on the adaptive process, which may contributes to remodeling induced bone quality reduction.

(4) Reduction of bone blood flow in nutrient vessel by long duration, repetitive, oscillatory fluid loading

In an attempt to evaluate the mechanism that intramedullary pressure stimuli can generate

pathological remodeling and lead to cortical lesion, we hypothesized that repetitive fluid flow stimulation can alter marrow cavity blood supply, which will induce partial ischemia through a reduction of nutrient blood supply. This can further trigger the pathologic remodeling in bone. Loading altered blood flow was evaluated in a turkey tibia model.

Experimental protocol: The animal was prepared as one-day terminal experiment on left tibia. Under the general isoflurane anesthesia, the left tibia of an adult, one year old male turkey was exposed at mid-shaft region. Two pins were inserted transcutaneously through the bone to prevent internal and external mechanical loading. The bone was drilled and tapped to provide an insertion of a specially designed fluid loading device allowing ImP oscillation in the marrow cavity. After carefully exposing the nutrient artery, a two-way ultrasonic Doppler probe was mounted to surround the artery close to the inlet entering the tibia. Two triple-rosette strain gauges were mounted on to the bone surface at mid-diaphyses. Dynamic volume flow and strains were measured and recorded during the entire loading protocol at which oscillatory ImP was applied in marrow cavity continuously for approximately 2.5 hours through various loading frequencies, i.e., 1 Hz, 3 Hz, 10 Hz, 20 Hz, and 30 Hz, and at various pressures, i.e., 15 mmHg, 50 mmHg, 76 mmHg, 100 mmHg, and 125 mmHg. Multiple measurements were performed for fluid pressure, strain, and nutrient blood flow. Data was collected by a pre-amplifier linked to an A/D converter and a computer (Dell PC). Our analog data acquisition card (model AT-MIO-16X, National Instruments, Austin, TX) collected the signals at sampling rate of 1000 Hz with 16-bit resolution for volume flow, ImP and strain.

Results: No strain greater than 1 με was observed during the dynamic fluid flow loading. Continuous repetitive fluid pressure decreased the volume flow in the nutrient vessel. The volume flow was reduced by 25 % of original volume after one hour of stimulation (Fig. 8). Even after 15 minutes of rest the full volume of blood did not return to the tibia.

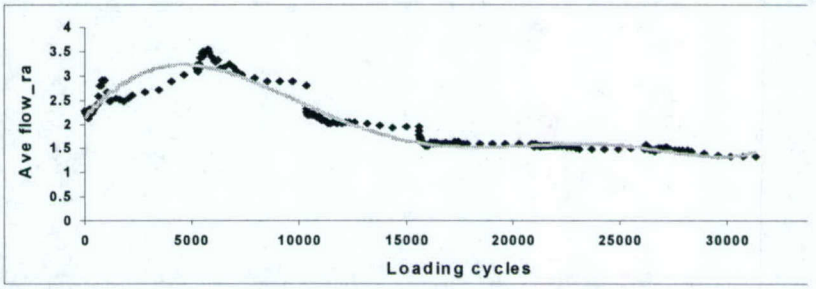

Fig. 8. Volume flow measured from the nutrient artery during ImP loading. Constant repetitive marrow fluid pressure resulted in the reduction of volume flow into the marrow cavity. Short term resting, e.g., 15 min, did not recover the nutrient blood flow to the original rate.

Discussion: These data suggest that the marrow blood supply can be substantially reduced by repetitive, long duration fluid pressure applied in bone. This reduction of flow could elucidate the adaptive response through partial ischemia of the nutrient blood supply of bone and sufficient cycle numbers. The mechanism may include partial closure of marrow vessels in response to the loading, which increases the resistance of the incoming blood flow, even if fluid flow was applied at a physiologic magnitude.

Implication of this work: The results imply that repetitive fluid flow loading may reduce nutritional blood supply and initiates ischemia of bone, which will trigger pathologic remodeling.

In summary, these results demonstrated that repetitive, high cycle, low magnitudes of fluid stimuli can initiate spatial fluid flow through bone and stimulate bone adaptation. At physiological magnitudes, ImP can increase and improve the perfusion process through increasing fluid pressure in the disuse conditions. Within the physiological range, new bone formation is proportional to applied fluid flow stimulation. While the pressure applied exceeds the physiological intensity or falls within the pathologic range, i.e., addition to normal physical loading, it may trigger extensive intracortical remodeling and thus weaken the quality of bone, i.e., increase intracortical porosity and induce lesions. This result suggests that repetitive cyclic

fluid loading in bone and marrow cavity has potentials to initiate blood vessel remodeling, e.g., nutrient vessel adaptation. Oscillatory ImP, if applied at the magnitude above the physiologic blood pressure will result in the reduction of the blood supply to bone, which can further result in pathological remodeling and weaken the bone mass

C. Key Research Accomplishments

- Long duration, repetitive oscillatory fluid loading is responsible to the nutrient vessel wall thickening, vessel smooth muscle cell proliferation and potentially reduces the blood supply in bone

  Repetitive dynamic cyclic ImP, whether at 3 Hz or 30 Hz, has shown influence on nutrient vessel remodeling inducing vessel wall thickening dependent on the loading cycle number, duration and pressure magnitude. This may potentially contribute to pathological remodeling in bone.

- Bone's remodeling activity is correlated to the magnitude of oscillatory fluid flow stimuli

  Fluid flow stimulation can significantly elucidate adaptive response if applied at proper fluid pressure and sufficient loading cycles. When fluid pressure applied exceeds the physiological intensity or is in the pathologic range, it can trigger increasing of intracortical porosity and an extensive remodeling process which may weaken the quality of bone.

- Trabecular bone turnover is sensitive to the number of cycle of fluid flow loading
  A high cycle number (elevated by loading frequency) of intramedullary fluid perturbation, if applied near physiologic magnitude, can significantly induce trabecular bone remodeling. Those bones loaded at the same cycle number, but at much smaller pressure amplitude, showed similar adaptive response.

- Long duration oscillatory fluid flow loading are responsible for the reduction of volume flow in nutrient vessel
  Marrow blood supply can be substantially reduced by repetitive, long duration fluid pressure applied in bone. This reduction of flow is potentially elucidating the adaptive response through partial ischemia of the nutrient blood supply of bone and sufficient cycle numbers. Short term rest can not recover the initial volume flow rate in the vessel. The results imply that repetitive fluid flow loading may reduce nutritional blood supply and initiates ischemia of bone, which will trigger pathologic remodeling.

D. Reportable outcomes

Qin, Y-X., Kaplan, T., Saldanha, A. and Rubin, C.T. (2003): Fluid Pressure Gradient, Arising from Oscillations in Intramedullary Pressure, is Correlated with the Formation of Bone and Inhibition of Intracortical Porosity. J Biomech, 36(10):1427-37.

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trabecular bone adaptation. Ann Am Soc Bone Mine Res, J Bone Min Res, 18:M158.
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Qin, Y-X., Kaplan, T. and Cute, M. (2003): Physiologic oscillatory fluid flow is responsible for bone formation and inhibition of bone resorption dependent on loading magnitude. 49th Ann Mtg Orth Res Soc. J Bone Min Res, 28:110.

E. Conclusions

Bone fluid flow is hypothesized to initiate aberrant remodeling which can ultimately compromise bone quantity and quality. Thus, pathologic response of load-induced fluid flow can potentially damage tissue viability, and initiate bone's remodeling process, ultimately leading to the stress fracture syndrome. Results from this year's study have shown that fluid flow stimuli in bone has potentials not only in initiating bone's remodeling, but also in vasculature adaptation. Within the physiological range, bone formation is proportional to applied fluid flow stimulation. While the pressure exceeds the physiological intensity or falls within the pathologic range, i.e., addition to normal physical loading, it triggers extensive remodeling and thus even weaken the quality of bone, i.e., increase intracortical porosity and induce lesions. Repetitive cyclic fluid flow in bone has shown an effect on the nutrient vessel remodeling inducing vessel wall thickening and smooth muscle cell proliferation, which may potentially contribute to pathological remodeling in bone. These results may provide insight of fluid flow in physiological and pathological remodeling and its role in stress fracture.

To understand the etiologic factors of stress fracture is extremely important. As a short-term goal, this study is aimed to acquire an improved understanding of the patho-physiology of stress fractures at the tissue level such as fluid flow alone triggers the osteopenia and lesion. As a long-range goal, if we can identify these osteogenic signals, this may help to design or alter specific training regimes to reduce the risk factors of stress fractures.

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Fluid pressure gradients, arising from oscillations in intramedullary pressure, is correlated with the formation of bone and inhibition of intracortical porosity

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Abstract

Fluid flow that arises from the functional loading of bone tissue has been proposed to be a critical regulator of skeletal mass and morphology. To test this hypothesis, the bone adaptive response to a physiological fluid stimulus, driven by low magnitude, high frequency oscillations of intramedullary pressure (ImP), were examined, in which fluid pressures were achieved without deforming the bone tissue. The ulnae of adult turkeys were functionally isolated via transverse epiphyseal osteotomies, and the adaptive response to four weeks of disuse ($n=5$) was compared to disuse plus 10 min per day of a physiological sinusoidal fluid pressure signal (60 mmHg, 20 Hz). Disuse alone resulted in significant bone loss ($5.7\pm1.9\%$, $p\leqslant0.05$), achieved by thinning the cortex via endosteal resorption and an increase in intracortical porosity. By also subjecting bone to oscillatory fluid flow, a significant increase in bone mass at the mid-diaphysis ($18.3\pm7.6\%$, $p<0.05$), was achieved by both periosteal and endosteal new bone formation. The spatial distribution of the transcortical fluid pressure gradients (∇P_r), a parameter closely related to fluid velocity and fluid shear stress, was quantified in 12 equal sectors across a section at the mid-diaphyses. A strong correlation was found between the ∇P_r and total new bone formation ($r=0.75$, $p=0.01$); and an inverse correlation ($r=-0.75$, $p=0.01$) observed between ∇P_r and the area of increased intracortical porosity, indicating that fluid flow signals were necessary to maintain bone mass and/or inhibit bone loss against the challenge of disuse. By generating this fluid flow in the absence of matrix strain, these data suggest that anabolic fluid movement plays a regulatory role in the modeling and remodeling process. While ImP increases uniformly in the marrow cavity, the distinct parameters of fluid flow vary substantially due to the geometry and ultrastructure of bone, which ultimately defines the spatial non-uniformity of the adaptive process.

Keywords: Bone fluid flow; Intramedullary pressure; Remodeling; Strain frequency; Osteoporosis; Strain; Stress; Adaptation; Fluid shear stress; Permeability

1. Introduction

Bone's ability to rapidly accommodate changes in its functional environment ensures that sufficient skeletal mass is appropriately placed to withstand the rigors of functional activity, an attribute described as Wolff's Law (Wolff, 1986). The premise of a mechanical influence on bone morphology, now a basic tenet of bone physiology (Lanyon and Baggott, 1976; Carter, 1982; Cowin, 1984; Martin and Burr, 1989; Frost, 1990; Goldstein et al., 1991), indicates that the removal of functional loading is permissive to the loss of bone (Donaldson et al., 1970; Rubin and Lanyon, 1987), while increased activity (e.g., exercise) will result in increased bone mass (Nilsson and Westlin, 1971; Jones et al., 1977; Krolner et al., 1983; Judex and Zernicke, 2000). Considering the strong anabolic potential of mechanical stimuli, and the devastating consequences of removing it, how the bone cell population perceives and responds to subtle changes in their functional environment remains a key issue in understanding the biological and biomechanical processes of bone remodeling. Further, identifying the regulatory components within the mechanical milieu may prove instrumental in devising a biomechanically based intervention for treating osteoporosis, accelerating fracture healing or promoting bony ingrowth.

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In addressing bone's adaptive response to mechanical stimuli, there are a number of different parameters used as the driving function for the optimization process, i.e., strain/stress magnitude, cycle number, number of events, strain tensor and strain energy density. The theories related to strain/stress based bone adaptation range from surface modeling as a function of strain magnitude (Fyhrie and Carter, 1986; Huiskes et al., 1987; Frost, 1990), to time-dependent modeling and remodeling (Beaupre et al., 1990). While there is an overall relationship between intensity of a stimulus and the magnitude of the response, there is very little evidence that the magnitudes of strains or stresses directly correlate to bone's morphological response (Brown et al., 1990; Gross et al., 1997).

It is also important to consider that bone is a highly structured composite material comprised of a collagen-hydroxyapatite matrix and a hierarchical network of lacunae-canaliculi channels. These tunnels permit interstitial flow of fluid through tiny microporosities (Piekarski and Munro, 1977; Weinbaum et al., 1994; Cowin et al., 1995; Cowin, 1999), and thus "by-products" of load, such as the change in fluid velocities or pressures, represent a means by which a physical signal could be translated to the cell (Pollack et al., 1977, 1984; Kelly et al., 1985; Montgomery et al., 1988; Reich et al., 1990; Rubin et al., 1997; Jacobs et al., 1998; Burger and Klein-Nulend, 1999).

To address the potential of this mechanism, load-induced bone fluid flow has been studied both theoretically and experimentally (Pollack et al., 1977, 1984; Gross and Williams, 1982; Montgomery et al., 1988; Reich et al., 1990; Dillaman et al., 1991; Zeng et al., 1994; Weinbaum et al., 1994; Hillsley and Frangos, 1994; Frangos et al., 1996; Jacobs et al., 1998; Tate et al., 1998; Weinbaum, 1998; Burger and Klein-Nulend, 1999; Weinbaum et al., 2001; Mak and Zhang, 2001). Despite the inevitably complex characteristics of fluid flow in porous media (e.g., time and pressure gradient dependent fluid movements), there is early experimental evidence that bone fluid flow driven by loading contributes to the adaptive response, particularly when it is coupled with strain magnitude as well as nutrition supply (Doty and Schofield, 1972; Kelly and Bronk, 1990; Kelly, 1996; Tate et al., 1998). While these experiments demonstrate that cyclic loading can generate significant bone fluid flow as evidenced by streaming potential measurements, there is little evidence to support fluid flow, as opposed to matrix strain, as the driving determinant in bone remodeling, especially under in vivo conditions. This scarcity of data may be associated with the inherent difficulty in separating bone fluid flow induced by mechanical loading from bone matrix strain, as fluid flow certainly is inevitably influenced by bone deformation. However, considering the anabolic potential of low magnitude, high frequency strain (Rubin et al., 2002), and the strong dependence of fluid flow on loading frequency (Qin et al., 1998; Weinbaum, 1998), it becomes essential to determine if these low-level signals derive some of their regulatory potential through fluid flow rather than matrix deformation.

In previous work, we have shown that intracortical fluid flow is induced not only by bone matrix deformation, but also by the intramedullary pressure (ImP) generated during loading (Qin et al., 2002). Further, we have shown that applying anabolic oscillatory ImP alone can induce transcortical fluid flow as measured by streaming potentials (Qin et al., 2000). The principal goal of this study was to test the hypothesis that bone fluid flow, in the absence of matrix strain, can serve as an anabolic stimulus to bone tissue. This goal was achieved by applying low level, high frequency fluctuations in intramedullary pressure in an avian model of disuse osteopenia.

2. Methods

2.1. Animals and experimental preparation

All surgical and experimental procedures were approved by the University's Lab Animal Use Committee. Under general halothane anesthesia, the left ulnae of ten adult, one year old, skeletally mature male turkeys were functionally isolated via transverse epiphyseal osteotomies (Qin et al., 1998). The metaphyseal ends of the ulna were covered with a pair of stainless steel caps and fully sealed with 6 ml of polymethylmethacrylate. Two Steinmann pins, 4 mm in diameter and 92 mm in length, were placed through the predrilled holes in the bone and cap unit. This preparation, including the internal caps, pins and external clamps, can prevent mechanical forces from being applied during daily activities, effectively serving to isolate the bone from any mechanical strain. A 4-mm diameter hole was drilled through the cortex at the dorsal side, approximately 1.2 cm from the proximal cap. The hole was tapped and a specially designed fluid loading device, with an internal fluid chamber approximately 0.6 cm^3 in volume, was firmly connected to the bone with an O-ring seal (Fig. 1). A diaphragm was included in the center of the chamber dividing the internal marrow fluid from the external oscillatory loading flow. A surgical plastic tube (2-mm inner diameter), which was connected to the device and passed through the skin, served to couple the device fluid chamber and the external fluid oscillatory loading unit. An injection plug (Terumo Medical Co., Elkton, MD) was connected to the external end of the tube to facilitate fluid flow loading. With the diaphragm and the injection plug, the bone marrow and oscillatory flow media were fully isolated

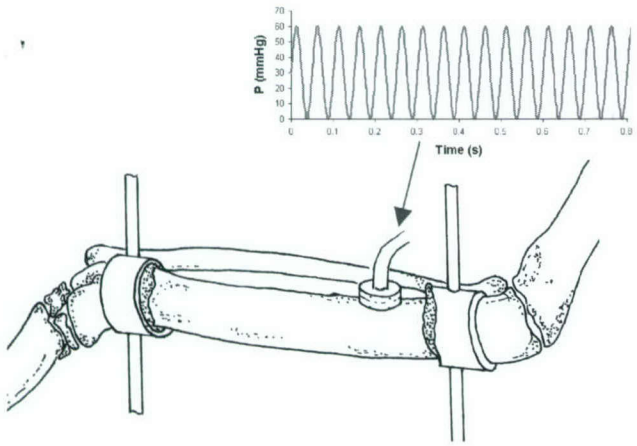

Fig. 1. A diagram of the functionally isolated turkey ulna preparation, such that oscillations in fluid flow can be achieved in the absence of matrix strain. External fixators on the two steel pins at two ends of ulna prevented external mechanical loads. A specially designed device connected into marrow cavity achieved oscillatory fluid loading. A low magnitude and high frequency (60 mmHg, 20 Hz) fluid pressure signal was imposed 10 min daily for 4 weeks in the disuse plus loading group, while the same procedures were prepared for disuse, yet the bone was subject to no exogenous fluid loading.

from the external environment to prevent any infection. To monitor the bone remodeling response, all animals were labeled weekly using tetracycline solution (15 mg-Kg^{-1}) through IV. The contralateral ulna served as control.

In addition to the 10 experimental animals, fluid flow was validated via two animals used to calibrate the loading device and its induced pressure magnitude with varied frequencies. The same surgical procedure was used in these animals. An additional tube was connected to the distal end of the ulna. Through this tube, a 50-psi pressure transducer (Entran EPX-10IW) was connected into the medullary canal, thus permitting measurement of the intramedullary pressure during animal rest and applied external ImP loading. The marrow pressures were recorded within the physiological pressure magnitude, $10 \sim 180$ mmHg, and at a variety of frequencies, $1 \sim 40$ Hz. The marrow pressure elevated by imposing ImP was then used to calibrate the loading system.

2.2. Dynamic fluid flow loading

The animals remained under close supervision until recovery from anesthesia and extensively monitored for an additional 2 h to make certain that they were able to stand normally and resume normal activities. Controlled fluid pressure oscillations started on day two after the surgical preparation. A sinusoidal fluid pressure was applied to the marrow cavity of the ulna at a peak magnitude of 60 mmHg at 20 Hz for 4 weeks ($N=5$). The remaining animals ($N=5$) were subject to an identical surgical procedure, except that there was no fluid pressure loading, and thus served to represent disuse.

2.3. Quantification of bone remodeling

Following a 10-min period of loading each day for 4 weeks, animals were euthanized via a bolus IV injection of saturated barbiturate. The ulnae of experimental and contralateral control were dissected free of soft tissue, and fixed for 48 h in 70% ethyl alcohol. After dehydration, the pair of ulnae for each animal was carefully positioned in a plastic box and embedded using polymethylmethacrylate.

2.3.1. Areal properties

Approximately 100 μm thick sections were cut from the midshaft of the ulnae using a precision diamond wire saw (Well Walter, Model 3241). Each section was microradiographed, scanned at a resolution of 600 dpi with a high-resolution film scanner (Minolta Dimage Scan Multi, Model F-3000, Japan), and converted to a binary image. The final image resolution was approximately 10 μm/pixel. The cortical area of each fluid loaded ulna was compared to the contralateral control ulna depending on the geometric similarity of the pair of the ulnae (Adams et al., 1995). Endosteal and periosteal new bone formation, as well as intracortical porosity, were traced using custom-written programs (PV-WAVE, Visual Numerics, Boulder, CO). Changes in bone mass, sites of new bone formation and porosity were determined by comparing the adapted area to the bone morphology of the contralateral control ulnae. Since the periosteal surface circumference remained unchanged in disuse bones, morphometric changes were determined by calculating areal differences between contralateral control and disuse alone in each animal. The initial starting point and the orientation of the sectors were based on the orientation of the ventral cortex in the ulna. This orientation is consistent with the turkey ulna anatomy.

In addition to total new bone formation, resorption and porosity changes were approximated using sector analysis in which the bone cross-section was divided into twelve equal angle (30°) pie sectors through the centroid of the bone section (Fig. 2) and compared to the animal's contralateral control. The number of sectors was selected by referring to previous studies, e.g., sectors ranged from 6 to 24 (Gross et al., 1997; Judex et al., 1997; Qin et al., 1998). The transcortical fluid pressure gradient was then calculated for each sector as the difference between fluid pressures at the endosteal and periosteal surfaces, where fluid pressure at the periosteal surface was considered zero. More specifically, the pressure gradient was estimated by dividing the averaged linear distance between the periosteal and endosteal surfaces using a total of 30 pairs of points in each

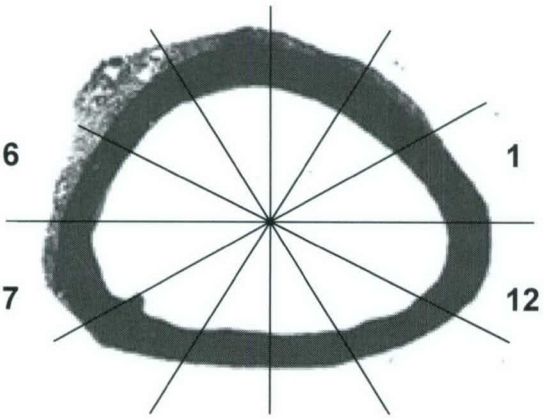

Fig. 2. A mid-diaphyseal cross-section is shown divided into 12 equi-angle sectors. Areal analyses were used to calculate surface bone mass changes and intracortical porosity in each sector. Bone mass changes were evaluated by comparing bone's adaptive responses between an experimental ulna and its contralateral control, as well as within and across experimental groups. An averaged transcortical pressure gradient was calculated in each sector by the pressure difference between endosteal and periosteal surfaces.

sector, where the relation can be expressed by the equation:

$$\nabla P_r = [\mathrm{ImP}]_{\mathrm{peak}} * [\text{cross cortical distance}]^{-1}$$

2.3.2. Histomorphometry

Quantification of the cortical modeling/remodeling response to these two distinct stimuli was determined using the distribution of the fluorochrome labels. Histomorphometric evaluation of undecalcified diaphyseal, metaphyseal and epiphyseal sections was performed on a Nikon Labophot system including epi-fluoresence microscopy, and reflected light microscopy. The fluorescent photomicrographs were taken through the microscope ($\times$ 10) and the photographs digitized at 600 dpi using a high resolution SONY digital CCD camera (Model DXC-950P, Japan). The final image resolution was approximately 1.1 μm/pixel. The image processing was performed using custom-written programs in PV-WAVE. Initial conditions were considered to be the labeling status of the contralateral control bone (Adams et al., 1995). The total area of new labeling and its transcortical distribution were then determined using imaging analysis in PV-WAVE. The magnitude and site specificity of disuse or disuse plus the oscillating fluid flow was determined by quantifying new bone formation at endosteal and periosteal surfaces, as well as porosities within the intracortical region.

2.3.3. Statistical analysis

Differences between disuse and disuse plus fluid flow were analyzed using paired student t-test. Significance was considered at $p \leqslant 0.05$. Linear regression was used to identify the relationship between the distributions of fluid parameters and the spatial modeling/remodeling parameters in bone in the fluid flow loading group using t-test of the linear regression (Watson, 1992). Thus, the significance of fluid flow on bone was tested in three ways: first, by determining the effective differences between experimental and contralateral control ulnae in both disuse and disuse plus fluid flow groups; second, by testing the significance between disuse and disuse plus fluid flow groups; and third, by correlation between fluid pressure gradient and site-specific response of adaptation in the fluid loading group. A paired two-sample student's t-test was performed to determine whether a sample's means were distinct from other criteria.

The contralateral controls in disuse and disuse plus fluid-loading groups served as an intra-animal control. Bone loss caused by disuse was evaluated by comparing experimental and contralateral control ulnae. The fluid-loading group had undergone the same surgical procedure and under the same experimental period as the disuse group. While intra-animal comparison is more accurate and more effective than cross animal comparison, the results of net bone adaptation, morphological loss or gain, were obtained from intra-animal paired data. The final results were explored between groups; between intra-animal experimental and control.

3. Results

3.1. Changes in areal properties

Morphometric changes in the group subject to disuse alone for 4 weeks indicated a significant loss of bone in the mid-diaphyseal cross sectional area; primarily due to an increase in the percent of the total bone envelope which was porotic ($5.7 \pm 1.9\%$, mean $\pm$ s.d.; total porosity vs. total bone area) as compared to that measured in the contralateral control ($1.6 \pm 0.7\%$; $p = 0.05$) (Fig. 3a,b). There was no evidence of bone resorption at the periosteal surface in any animal. Total area of bone mass (including the area of porosity) in the animals subject to 4-week disuse remained similar between disuse and contralateral control, yielding total cross-sectional areas of $53.8 \pm 3.5\,\mathrm{mm}^2$ (mean $\pm$ s.d.) and $52.7 \pm 2.5\,\mathrm{mm}^2$, respectively. Morphometric analysis showed that 10-min per day of the oscillating fluid flow resulted in a significant increase in bone mass at the mid-diaphysis ($18.3 \pm 7.6\%$; total new bone/total bone area) ($p < 0.05$), primarily due to periosteal ($16.1 \pm 6.5\%$, $p < 0.05$), as opposed to endosteal ($2.2 \pm 1.6\%$, $p = 0.13$) new bone formation (Fig. 3c).

The sites of modeling and remodeling response in bone were consistent using microradiograph and

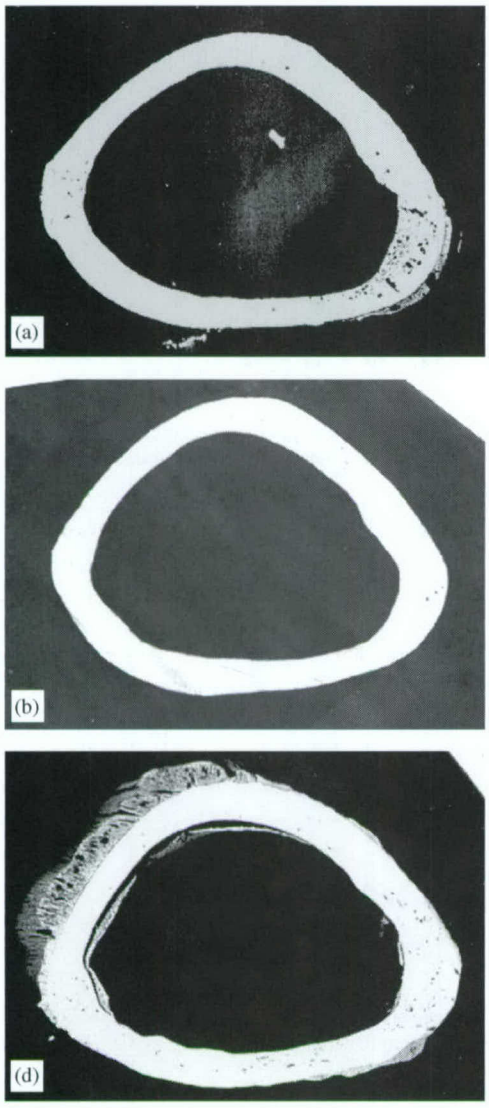

Fig. 3. Microradiographs of (a) animal subject to 4-week disuse resulted in significant bone loss by increase of intracortical porosity. (b) contralateral control of disuse ulna (a). (c) 4-week fluid flow loading resulted in significant new bone formation in periosteal and endosteal surfaces, yielding total of 18% new bone formation as compared to control.

fluorescent labeling analyses, in which new bone formation and intracortical remodeling were identical at the locations of the adaptive response.

3.2. Sector specific stimulation of bone adaptation

In animals subject to the fluid pressure oscillations, despite a uniform marrow pressure at the endosteal surface, oscillatory ImP generated non-uniform spatial distributions of transcortical fluid pressure gradients through the cortex, rising 37% from $4.9 \pm 0.2\,\text{kPa-mm}^{-1}$ in sectors 1 & 7, to $6.7 \pm 0.4\,\text{kPa-mm}^{-1}$ in sectors 5 & 6 (Fig. 4).

Although the ImP stimulus was ultimately non-uniform about the cortex, the non-uniform patterns were consistent between animals. At the periosteal surface, maximum new bone formation was observed in sectors 4, 5 and 6, yielding new bone formation of $1.7 \pm 0.6\,\text{mm}^2$, $3.1 \pm 1.0\,\text{mm}^2$ (25% & 41% gains, respectively), and $2.8 \pm 1.2\,\text{mm}^2$ (39% gain) ($p < 0.02$), respectively (Fig. 5). The area of endosteal new bone gain in each sector showed an average gain of $0.13 \pm 0.08\,\text{mm}^2$ ($p = 0.13$), ranging from a maximum gain of $0.5 \pm 0.3\,\text{mm}^2$ (7.8% gain in sector) in sector 4, to zero change in sectors 1 & 8. There was no significant difference of endosteal new bone formation among sectors. Oscillatory ImP stimuli resulted in increase of porosity area in each sector from maximum increase of $0.37 \pm 0.12\,\text{mm}^2$ (6.3%) in sector 12, to a minimum increase of $0.17 \pm 0.05\,\text{mm}^2$ in sector 5 (Fig. 5).

3.3. Correlation between fluid pressure gradient and bone formation

A strong correlation was observed between the transcortical fluid pressure gradient, ∇P_r, induced by oscillatory ImP and periosteal new bone formation ($r = 0.77$, $p = 0.01$), as well as total new bone formation ($12.1\,\text{mm}^2$ gain with 18.3% increase, $p \leqslant 0.01$) (Figs. 4 and 6). Endosteal new bone formation was weakly correlated with the ∇P_r ($r = 0.53$, $p = 0.08$). Interestingly, a negative correlation ($r = -0.75$, $p = 0.01$) was found between increased area of intracortical porosity and ∇P_r (Fig. 6).

4. Discussion

To determine the regulatory influence of bone fluid flow on the adaptive response of bone, it is necessary to gather both in vitro and in vivo data from a variety of fluid flow conditions. In the past few years, many studies report proliferative responses of osteoblast-like cells, and inhibiting effects on osteoclast-like cells, under pulsing or oscillatory fluid flow loading in culture (Frangos et al., 1996; Rubin et al., 1997; Jacobs et al., 1998; Burger and Klein-Nulend, 1999). For example, oscillatory flow resulted in greater cellular responses than steady flow (Jacobs et al., 1998). These studies unveiled a cellular response to fluid flow loading in an in vitro environment. However, it is possible that the fluid magnitudes examined in these studies were not entirely consistent with those levels which are a fluid representative of physiological levels, e.g., at relatively high pressure magnitudes and at great fluid shear stresses. Further, many studies use immature cells or cells from very young animals, which may not reflect bone cell behavior which could be expected in adults under the conditions of normal mature cell. Finally, it is

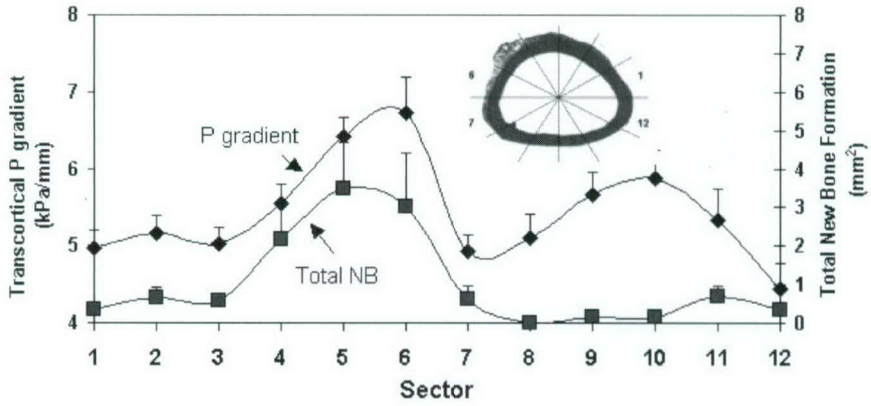

Fig. 4. Mean value (±s.e.) of transcortical fluid pressure gradient distributions for each of 12 sectors in the animals subject to fluid flow loading. Maximum pressure gradient was observed in sector 6, which corresponded to the site of maximal new bone formation. Minimum pressure gradients were located corresponding to least new bone formation sectors, i.e., sector 12 & 7.

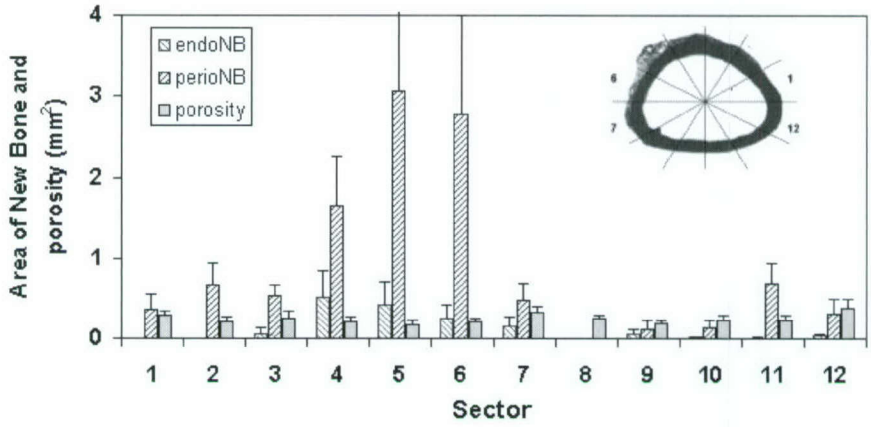

Fig. 5. Spatial distributions, mean (±s.e.), of new bone formation at periosteal and endosteal surfaces as well as an increase in intracortical porosity in each sector. ImP resulted in significantly new bone formation, achieved primarily by periosteal new bone formation ($p<0.02$). Fluid flow loading also minimized intracortical porosity.

important to consider that in vitro experiments, while proving insight into the mechanism of a cellular response, do not ultimately indicate whether new bone will be formed, or that bone loss can be inhibited. This suggests that knowledge of how bone cells accommodate a systematic, physiologic and morphologically "appropriate" fluid flow environment is important to address the relevance of these signals in controlling bone's adaptive response. In an attempt to examine the anabolic potential of fluid flow loading in vivo, this study applied oscillatory intramedullary fluid pressure at low magnitude and high frequency, where it was found to stimulate bone formation and reduce bone porosities caused by disuse. This flow stimulus is considered to be physiological in the in vivo flow environment. Thus, if fluid flow is "sufficient," it is capable of stimulating new bone formation and inhibition of bone resorption with only a daily 10 min period of loading. This implies that physiological fluid flow is indeed a mediator critically involved in bone modeling and remodeling, and that its influence can be realized in the absence of matrix strain per se.

Given the porous nature of bone, the fluid filled spaces invariably generate a flow upon mechanical loading. In general, load-induced flow and its associated matrix strain are usually coupled. Therefore, segregating the regulatory potential of matrix strain from the anabolic potential of fluid flow becomes inherently difficult. Matrix strain, as a general parameter of bone receiving mechanical loading, is commonly used in describing bone tissue deformation. If bone fluid flow is indeed a key mediator for bone modeling and remodeling, then it is important to test the accommodation of tissues and cells to a customary flow loading environment. This study tried to separate matrix strain and convective fluid flow by dynamically pressurizing the marrow cavity which drives interstitial fluid to flow. The fluid magnitude for such a flow remained in the physiological range generated in the marrow cavity by an animal's normal activities (Fritton et al., 2000).

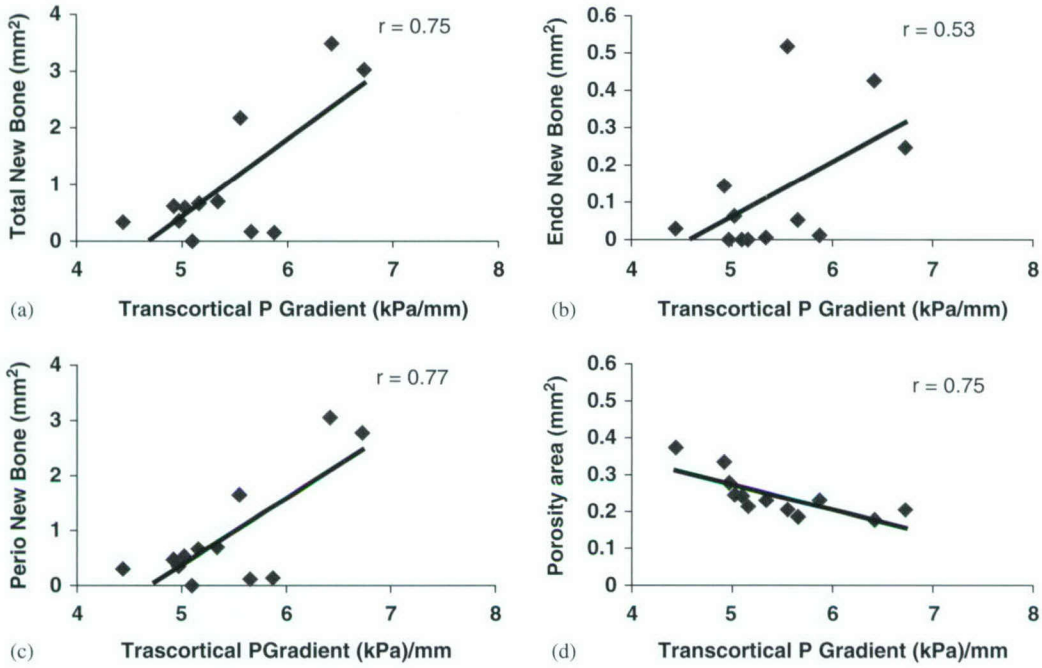

Fig. 6. The strong correlation between calculated pressure gradients and total new bone ($r=0.75$, $p<0.02$) (a). While a weak correlation was observed between endosteal new bone formation and pressure gradients ($r=0.53$) (b), a strong correlation was observed between transcortical fluid pressure gradients and periosteal areal adaptation ($r=0.77$, $p<0.02$) (c). A negative correlation was identified between increase in intracortical porosity and increase of transcortical pressure gradients ($r=0.75$, $p<0.02$) (d).

When dynamic hydraulic pressure is pressurized in the marrow cavity and interstitial pore space, however, there was concern that the imposed ImP would create deformation in the matrix. Assuming a solid material modulus of 10 GPa and isotropic elastic mechanical behavior of cortical bone, it is estimated that a maximum fluid pressure on the order of 8 kPa will result in approximately 0.8 με in the matrix. Further, assuming a strain–stress relation in a poroelastic model and a bulk modulus of 5 GPa for the two-phase material, then the calculated matrix strain falls to less than 0.1 microstrain (Neidlinger-Wilke et al., 1994; Cowin, 1999). It is difficult to envision a physical mechanism by which ImP loading resulted in new bone formation could be generated by such small matrix strain, particularly in light of the strong in vitro evidence that fluid flow can perturb to the biological response of bone cells. This experiment suggests that fluid flow can, in and of itself, influence parameters of bone formation and resorption.

The sites of greatest osteogenic response correlated with the greatest gradient of transcortical fluid pressures. The strong correlation between new bone formation and fluid flow suggests that fluid components, i.e., pressure gradients, a close source driving fluid velocity and fluid shear stress, may directly influence the response of bone cells to mechanical stimulation. In addition, the correlation between minimal intracortical porosity and elevated fluid pressure gradients implies that a basal level of convectional bone fluid flow is critical in preserving cortical mass against disuse, such as conditions of bed rest and microgravity. At the very least, it is clear that extremely low-level perturbations of fluid flow, as induced by high frequency oscillations, are providing necessary signals to inhibit intracortical porosity and stimulate new bone formation. Given the anabolic potential of these high frequency signals (Rubin et al., 2002), and the rapid rise in fluid velocities that occur because of high frequencies even in conditions of very low strain (Weinbaum, 1998; Weinbaum et al., 2001), it is certainly possible that signaling the cells responsible for orchestrating bone adaptation is achieved not by subtle changes in matrix strain, but by changes in fluid flow.

The implication of the strong correlation between fluid flow components, i.e., pressure gradients, driven by ImP, is interesting because of its potential to impose fluid shear stress in the cellular environment. A number of theoretical models have been proposed to describe a potential mechanism of fluid pressure and fluid shear stress in bone (Dillaman et al., 1991; Weinbaum et al., 1994; Cowin et al., 1995; Mak et al., 2000), which have been supported by mounting in vitro experimental work (Frangos et al., 1996; Jacobs et al., 1998; Burger and Klein-Nulend, 1999). The effects of an increase in fluid flow induced by oscillatory ImP can potentially influence bone cell activities through several coupling mechanisms. First, raising the ImP can result in a

corresponding increase of outward fluid flow through various fluid pathways, which include the vascular system and the extensive lacunar–canalicular spaces in which the bone cell population resides. Increased fluid velocities can produce fluid shear stresses on the endothelial lining cells of vessels (Girard and Nerem, 1995) and on the bone cells in the lacunar spaces (Weinbaum et al., 1994), where the oscillatory ImP can alter the fluid shear stresses on the cell population and trigger a cellular response. Second, a nutrient pathway for metabolism and the proper disposal of waste products generated from catabolic activities occur through fluid channels. In the soft tissue, molecular diffusion is considered the major pathway for transportation of metabolites (Otter et al., 1999). Because of the relatively dense structure of cortical bone, however, the diffusive mechanism may, in fact, be insufficient to play an adequate role in transporting metabolic constituents between osteocytes and the surrounding vascular canals. An imposed dynamic ImP will enhance this fluid transportation from the blood supply to osteocytes through this convective perfusion mechanism (Piekarski and Munro, 1977; Tate et al., 1998; Wang et al., 2000), where the greatest exchange occurs at sites of greatest pressure gradients.

The fluid magnitude for ImP stimulation was imposed at physiological levels. Via Haversian canals, fluid flow can be modulated by blood flow during ImP stimulation. Blood supply to the long bone is achieved through both marrow and periosteal nutrient vessels, with the main vessel dividing before entering the marrow cavity. The blood supply via the marrow is primarily, but not entirely, centrifugal (Brookes, 1967; Singh and Brookes, 1971; Slater et al., 1991; Churchill et al., 1992; Kiaer, 1994; Bridgeman and Brookes, 1996). The marrow pressure caused solely by the circulation is on the order of 10 to 50 mmHg, depending on the species (Kumar et al., 1979; Bryant, 1983; Otter et al., 1999), and is approximately 18 mmHg in the turkey ulna. While mechanical fading can increase marrow pressure on the order of 150 mmHg with $800 \sim 1000$ microstrain axial loading, ImP oscillations which can attain pressures of 60 mmHg, would incorporate with the circulatory blood pressure and impact on bone fluid flow. Enhanced fluid convection dependent on either transcortical flows through particular fluid perfusion pathways or altering basal blood flow is supported by the non-uniformity of the surface bone formation.

While physiologic fluid flow showed the potential to initiate the modeling and remodeling process, dynamic components of this fluid may also play an important role in the regulation of adaptation. It is recognized that bone tissues respond very differently to static vs. dynamic load environments, and results in an adapted structure which demonstrates similar peak strain magnitudes during vigorous activity (Lanyon and Rubin, 1984). These regulatory "temporal" components may include strain rate, strain frequency, and strain gradients (O'Connor et al., 1982; Rubin and McLeod, 1994; Turner et al., 1995; Gross et al., 1997; Qin et al., 1998). These temporal components result not only in local matrix deformation, but also in fluid flow, streaming potentials, and other physical phenomena, which also influence cell responses. For example, in the case of the turkey ulna, 10 min of loading per day at 1 Hz requires a peak induced longitudinal normal strain greater than 700 $\mu\varepsilon$ to maintain bone mass, while a relatively high frequency (30 Hz) loading regimen reduces this threshold to 70 $\mu\varepsilon$ (Qin et al., 1998). The stimulatory effects of fluid flow, driven at physiological magnitude and high frequency but with minimal matrix strain, may depend on the cellular response due to (1) intermittent rather than static flow constant velocity, (2) direct fluid shear stress perturbation, (3) the cumulative effect of small local fluid movements resulting in cells accommodating to large flow cycles, and (4) even an "amplified" effect on the bone cell which could result in pressurization and/or fluid shear stress on the cell (Weinbaum et al., 2001). Again, these data, while not intended to diminish the role of bone strain, imply that anabolic fluid flows, applied in a dynamic manner, can have a tremendous influence on bone mass and morphology even under conditions of extremely low matrix deformations.

That fluid flow results in periosteal expansion in response to intramedullary pressure and transcortical pressure gradients help identify a physical mechanism for the response. Since the periosteum is often referred to as an impermeable layer for fluid perfusion, it is understandable that periosteal modeling requires fluid exchange and/or flow to initiate such an adaptive process. Fluid flow resulted in periosteal bone formation in this study, and thus implies that oscillations of ImP influence bone fluid perfusion and convection in many ways. While the endosteal surface provides an open circulation between marrow pressure and intracortical flow, the interstitial fluid flow in bone must flow out of the mineral to the periosteal surface through a variety of fluid pathways (Morris et al., 1982; Tate et al., 1998; Wang et al., 2000). Since the loading pattern used in these experiments was oscillatory, it may not be necessary that fluid physically flowed out of the periosteal surface but, instead, the oscillation itself may serve as a stimulatory signal. Under oscillatory fluid stimulation, however, a local fluid pressure gradient may be built up with the semi-permeable periosteal boundary condition which will create a flow at the periosteal surface. The spatial distributions of such fluid flow patterns ultimately is dependent on the fluid pressure gradients, defined somewhat by the geometry, ultrastructure and fluid pathways of the bone.

Fluid pressure gradients were calculated based on the assumption of zero pressure magnitude at the periosteal

surface boundary. Using a poroelastic two-phase FEA model, we have previously calculated that transcortical fluid pressure gradients at the periosteal surface were relatively similar when considering either impermeable or semi-permeable surface boundary conditions (Qin et al., 2000). In this particular case, the calculated transcortical pressure gradients may remain proportional regardless of the periosteal permeable conditions, e.g., impermeable vs. semi-permeable. This suggests that the correlation between the morphometric bone formation and the calculated transcortical fluid pressure gradients may be consistent for different permeabilities of the periosteum. However, to better understand interstitial fluid movement, identifying the hydrostatic permeability of cortical bone and the surface boundary conditions are indeed critical. It was found that bone permeability was dependent on many factors, i.e., age and spatial location. Using a canine tibia model, the permeability of puppy tibiae is six times higher than that of adult tibiae (Li et al., 1987). The high permeability of their cortical bone may explain the increase in periosteal new bone formation seen in puppies when a venous tourniquet is applied. While the endosteum is permeable, they have found that the periosteum is, in essence, impermeable unless the periosteal superficial layer is removed in the adult canine tibial cortex. Many tracer studies have indicated that fluid perfusion can penetrate both endosteum and periosteum. Penetration of bone fluid can be greatly enhanced by convection through mechanical loading. It was observed that, in loaded bone, the concentration of tracer dispersed through the mid-diaphysis and surface of the cortex was significantly higher than that which was measured in the unloaded bone (Tate et al., 1998). Nevertheless, to identify the periosteal permeability in this model will help to understand the fluid flow pattern in bone through the convection mechanism. This may be important for future work.

There were several other limitations in the study. Like many in vivo studies, this protocol required an invasive surgical preparation, which potentially altered the bone metabolism and introduced complications during fluid loading. To minimize the variability that inevitably occurs in a biological system, the influence of the surgical procedure might, to a certain degree, be determined by the sham group and the contralateral control. In addition, the fluid loading hole was located on the dorsal side of the ulna. The hole was physically 3 cm away from the midshaft where the cross sectional morphometric analysis was performed. This distance exceeds the threshold of a surgical procedure inducing bone modeling/remodeling activity. In addition, there were distinct limitations in relying on the analytical analysis, in which it is necessary to simplify what is undoubtedly a complex biological system. There are several fluid filled spaces in the cortical structure, e.g., Haversian channel, lacuna-canaliculi, and micropore space. The calculation of fluid pressure gradients was based on the assumption of uniform distributions of these porosities in bone. Although accurately determining the true distributions of fluid channels and porosity of cortical bone is immensely difficult, the potential inaccuracy can be modified with further determined spatial distribution of pore size and permeabilities.

In conclusion, in an effort to determine how bone tissue senses fluid flow related stimuli, and their importance to the adaptive response in bone, we have developed an animal model which can induce oscillatory fluid flow in the absence of bone matrix deformation. The results indicate that small perturbations of basal fluid flow can influence both bone formation and resorption. New bone formation on the periosteal surface was strongly correlated to fluid pressure gradients, suggesting the adaptive response to be influenced by both fluid velocity and shear force. These data also suggest that maintaining anabolic cortical fluid flow is essential to maintain intracortical bone mass against the effects of disuse. The results imply that the fluid flow induced by physiological values is essential and important in retaining bone quality and quantity, and that small fluctuations in fluid flow, achieved via pressure differentials, has potential for therapeutic applications against skeletal disorders even in the absence of mechanical strain.

Acknowledgements

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DOES CYCLIC INTRAMEDULLARY FLUID FLOW LOADING INDUCE NUTRIENT BLOOD VESSEL ADAPTATION?

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INTRODUCTION:

The dynamics of arterial vessel adaptation is a crucial subject in understanding the pathological remodeling in bone tissue, which likely contributes to skeletal diseases, e.g., stress fracture. Stress fractures, occurring commonly in military soldiers and athletes trainings, are the result of multiple microdamages induced from repetitive cyclic activities. It is hypothesized that stress fractures are initiated by bone remodeling and catalyzed by pathologic bone fluid flow.

Arterial vessel wall adaptation to acute or chronic flow changes is proposed to be responded to fluid shear stress at the endothelium. Such vessel adaptation in the nutrient artery of bone can be potentially induced by mechanical load generated flow. The objective for this *in vivo* study is to determine the interrelationship between the cyclic hydraulic stimulation in the marrow cavity and the adaptive response of nutrient blood vessels.

METHODS:

Surgerial Preparation. Under anesthesia, the left ulnae of 17 adult, one year old male turkeys were operated. A 3-mm in diameter hole was drilled and tapped near the proximal end of each ulna, allowing the insertion of special designed fluid loading device. Following the same procedure as the left, a sham device was placed into the contralateral right ulna as a sham control.

In vivo Loading Treatment. The high repetitive intramedullary pressure was accomplished through a special designed oscillation system, which can be controlled to produce pressure with varied frequency and magnitude. A sinusoidal fluid pressure was applied to the ulnae, 10 minutes per day, with the magnitude of 76-90mmHg at 3Hz for 3 weeks (n=4), 76mmHg at 30Hz for 2 weeks (n=4), 76mmHg at 30Hz for 3 weeks (n=6), and 50mmHg at 30Hz for 4 weeks (n=3).

Histomorphometry Analysis. After the animals were sacrificed, the ulnae were extracted and their nutrient blood vessels were dissected, cleaned, and fixed in 10% formalin solution. The adaptive responses of the nutrient vessels were analyzed through a standard soft tissue histology procedure, which included embedding in paraffin wax, sectioning to produce approximately 5-10μm thin slices, and staining with hematoxin and eosin. The histomorphometry of the vessels were examined using a digitized microscope, and the cross-sectional images, e.g. fig. 1, were analyzed using custom-written software to calculate the area of the vessel wall. Student t-test was used to evaluate the significant between the experimental and the control groups.

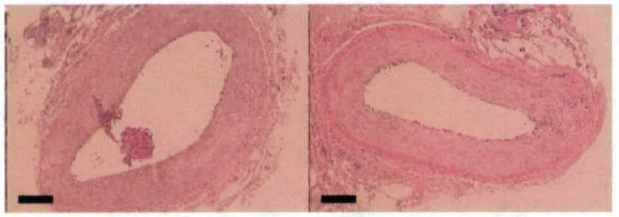

Fig. 1. Cross-sectional images of the nutrient blood vessels, left: control; right: loaded. (-- is 100μm)

RESULTS:

Several comparisons were intended and observed through the four animal groups, 1) the vessels subject to high repetitive fluid loading to the sham controls within each animal group 2) nutrient vessel wall adaptation with varied fluid loading frequencies 3) nutrient vessel wall adaptation with increased duration of loading.

Our preliminary data indicate that the cross-sectional vessel wall area increased 23%, 6%, 32%, and 97%, at the loading side, respectively. (fig. 2 & 3). The areal changes of the vessel with varied frequencies (3Hz and 30Hz) and same duration (3-weeks), do not show any significant difference, $p<.75$ (fig. 2). However, the areal changes has demonstrated increasing as the duration of loading increased, i.e., vessel area for the 3-weeks of loading is 5.7% higher than the 2 weeks, and the area for the 4-weeks loading is approximately 31% higher than the 3-weeks and 38% higher than the 2-weeks (fig. 3). Interestingly, the control groups also showed a slightly decrease in vessel wall area. This might be a result of decelerating general birds' activities as the experiment progress.

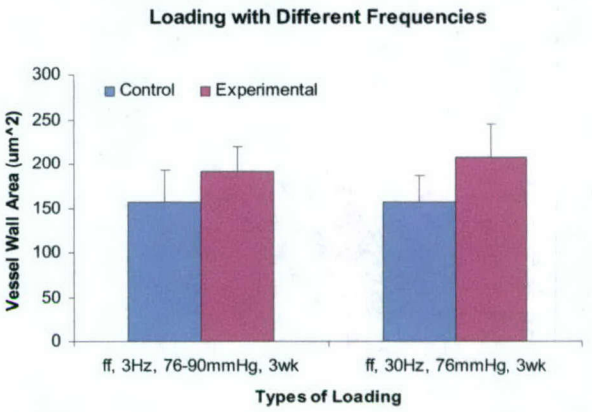

Fig. 2. Histomorphometry of the nutrient vessel cross sections analyzed from ulnae subjected to high repetitive fluid flow (ff) at 3 and 30Hz, 10 min per day for 3 weeks. Average vessel wall area showed no significant difference with increase frequency ff.

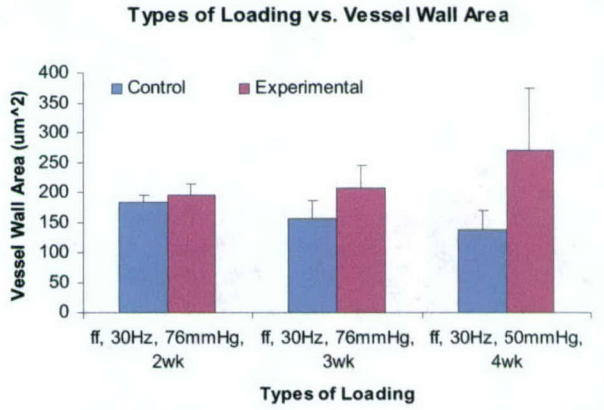

Fig. 3. Histomorphometry of the nutrient vessel cross sections analyzed from ulnae subjected to high repetitive fluid flow (ff), 10 min per day, at 30Hz, 76mmHg for 2- and 3-weeks, and 30Hz, 50mmHg for 4-weeks,. Average vessel wall area showed an increase with increased durations.

DISCUSSION:

These preliminary results strongly indicated that cyclic fluid loading in bone does act on the nutrient blood vessel morphological adaptation. Yet, it seems that certain parameters might have a higher impact in manipulating nutrient vessel morphology. For instance, the duration of loading may have a higher potential in triggering the vessel wall adaptation, while the increases in frequencies might has lesser effect. Nevertheless, this implies that vessel adaptation, induced by high repetitive fluid loading, could future reduce the blood supply to bone and potentially generate pathological remodeling in bone tissue, which may contribute to stress fracture. Further analyses will be performed to gain more insight on how fluid components affect vessel morphologies at both tissue level, i.e., area changes, and cellular level, i.e. endothelial and smooth muscle cells orientations and regulations.

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Alteration of long duration repetitive vascular fluid flow as a potential etiology factor of stress fracture

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INTRODUCTION: It is generally agreed that stress fractures are caused by overuse of skeletal tissue, and that the skeleton fails to adapt quickly enough to new loading conditions imposed on it. However, the specific etiology of stress fracture is the subject of great controversy. Stress fractures most often occurring before accumulation of materials damage could occur and they occur in cortical locations of low, not high strain. Intramedullary pressures, which significantly exceeding peak arterial pressure generated by prolonged, strenuous exercise, can alter the blood flow into the medullary canal and hence to the inner 2/3 of the cortex. We hypothesize the load-induced alteration of bone vascular flow supply in nutrient artery as a potential etiology factor of stress fracture. The flow rate change in the nutrient artery responding induced medullary pressure is investigated.

METHODS: In vivo experiment is performed using an avian tibia model (N=4). Nutrient artery was located at the area of tebia fibula fusion and the probe of flow rate meter (TS420, Transonic System Inc.) was hooked up to it. A specially designed fluid loading device with an inside diaphragm was connected to the medullary cavity. Control side nutrient artery was also exposed to hook the probe under the same procedure. The pulsatile medullary fluid pressure was applied via a special designed system with controlled frequency and magnitude at the experimental side. And at the same time, the real-time blood flow rate inside nutrient artery is recorded via the flow rate meter. Each fluid loading bout including a group of frequencies selected at 1Hz, 1.5Hz, 2Hz, 5Hz, 10Hz and 30Hz. In each loading frequency, 5 loading amplitudes, from 0.5V to 2.5V with 0.5V interval, were consequently applied and each lasted for 20 second. Average flow rate in nutrient artery was calculated for each 20 seconds loading from both experimental and control side. Normalized blood flow rate was plotted according to time sequence and loading cycles.

RESULTS: It has been demonstrated that blood flow rate inside nutrient artery changed according to time and loading cycles (Fig. 1). Initial loading resuted in flow rate increasing (~ 5000 cycles). However, repetitive loading above 5000 cycles resulted in continuously decrese of flow rate (5000 to 30000 cycles), yielding approximately 100% reduction of peak flow rate. Short period of resting (10~20 min) from loading could not fully recover the blood flow rate.

DISCUSSION: As the main blood vessel that penetrates the bone cortex and then ramified into bone tissue, nutrient artery provides almost 2/3 of the nutrient supply needed by the bone. Reduction of the blood flow rate in nutrient artery directly lead to the reduction of bone nutrient supply. From our study, blood flow is depressed by the long duration repetitive fluid pressure resulted from loading and will certainly compromise the bone tissue quality, due to the prolonged, repetitive loading. Our study also shows that nutritional flow rate can not be fully recovered by a short period of resting, e.g., 10 min, between loads. The reason could be the time of rest is not long enough, or the ratio between loading and resting is not optimized for the fully recovery of flow rate. This loading-resting-loading pattern can be regarded as exercising-resting-exercising pattern in the reality, further experiments are needed to address this question because it may be the key to find optimized training pattern and reduce the incidence of stress fracture.

CONCLUSION: This work suggests that reduced vascular flow supply as a potential etiology factor of stress fracture. The mechanism is by compromising bone remodeling procedure to finally lead to stress fracture. Future work will be directed to the fully understanding of the detail mechanism and consequence of the nutrient flow reduction.

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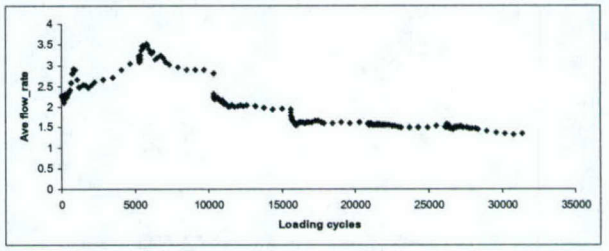

Fig.1. Typical flow rate change with increasing of loading cycles and loading times.